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Using chemical, microbial and fluorescence techniques to understand contaminant sources and pathways to wetlands in a conservation site

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Abstract

Nutrients and faecal contaminants can enter wetland systems in a number of ways, with both biological and potentially human-health implications. In this study we used a combination of inorganic chemistry, dissolved organic matter (DOM) fluorescence and *Escherichia coli* and total coliform (TC) count techniques to study the sources and multiple pathways of contamination affecting a designated sand dune site of international conservation importance, surrounded by agricultural land. Analysis of stream samples, groundwater and dune slack wetlands revealed multiple input pathways. These included riverbank seepage, runoff events and percolation of nutrients from adjacent pasture into the groundwater, as well as some on-site sources. The combined techniques showed that off-site nutrient inputs into the sand dune system were primarily from fertilisers, revealed by high nitrate concentrations, and relatively low tryptophan-like fulvic-like ratios < 0.4 Raman units (R.U.). The *E. coli* and TC counts recorded across the site confirm a relatively minor source of bacterial and nutrient inputs from on-site grazers. Attenuation of the nutrient concentrations in streams, in groundwater and in run-off inputs occurs within the site, restoring healthier groundwater nutrient concentrations showing that contaminant filtration by the sand dunes provides a valuable ecosystem service. However, previous studies show that this input of nutrients has a clear adverse ecological impact.

Keywords: Faecal indicator bacteria; dissolved organic matter fluorescence; water chemistry; groundwater; sand dunes; dune slacks; ecosystem service.

Introduction

The global availability and mobility of nitrogen has increased rapidly over the past five decades (Galloway and Cowling, 2002) and the damaging impacts it has on freshwater ecosystems are widely documented (Camargo and Alonso, 2006). Aquatic systems are extremely sensitive to nitrogen and are threatened by atmospheric deposition inputs (Fowler et al., 2005) as well as point sources and diffuse sources which can enter aquatic systems via numerous pathways such as through runoff, streams and groundwater. Tracing sources of aquatic pollution is therefore often problematic (Withers et al., 2009).

Within rural areas river water quality (Hooda et al., 2000) and groundwater quality (Oakes et al., 1981) are primarily impacted by agricultural diffuse pollution (Novotny, 1999). Atmospheric nutrients have been demonstrated to have adverse impacts on the ecology of protected dune habitats (Jones et al.,

2013, Plassmann et al., 2008, Field et al., 2014). However, the specific impacts of relatively low levels of nutrients from groundwater on aquatic habitats in dune systems have only recently been documented (Rhymes et al. 2014). As well as nutrients, diffuse inputs of dissolved organic matter (DOM), and micro-organisms into groundwater and surface waters also occur via runoff, field drainage and leaching, as a result of agricultural practices such as slurry and fertiliser application. Previously, these diffuse inputs have largely been characterised by using nutrients as a proxy (e.g. Vadas et al., 2007), although more recent studies are now examining diffuse sources and pathways by investigating pathogenic micro-organisms (Kay et al., 2008) and characterising DOM by natural fluorescence (Hudson et al., 2007). To date there have been no studies combining chemical, fluorescent and microbial techniques to help decipher multiple diffuse sources and pathways. Excitation emission matrix fluorescence spectroscopy (EEMS) can be used to trace DOM from agricultural sources (Baker, 2002, Old et al., 2012). EEMS is sensitive enough to characterise fulvic-like, humic-like and protein-like substances (Tryptophan-like and tyrosine-like) within the DOM to help characterise and quantify the extent of contamination by effluents from different sources (Hudson et al., 2007). Fulvic-like and humic-like substances are derived from the breakdown of plant material (Stedmon et al., 2003), whereas large inputs of tryptophan-like substances are associated with readily biodegradable material from sewage and farm waste slurry (e.g. Baker, 2001). Agricultural diffuse sources such as animal waste are characterised by high protein-like fluorescence with very high ratios of tryptophan-like to fulvic/humic-like fluorescence compared to stream waters (Baker, 2002), these ratios are sensitive enough to characterise inputs from different livestock animals such as pigs and sheep (Baker, 2002).

Currently, the WHO Guidelines for Drinking Water Quality, adopted as standard in many countries, use total coliforms (TC), or specifically *Escherichia coli* (*E. coli*) a sub group of faecal coliforms, as faecal indicators for the safety of water supplies. In some countries, such as The Netherlands and Denmark, groundwater is abstracted from sand dune systems to supply drinking water, indicating the importance of understanding the fate and occurrence of TC and *E. coli* within these systems (Smeets et al., 2009). The enumeration of TC and *E. coli* is also used as an indicator of water quality within the revised bathing water directive; *E. coli* counts greater than 10,000 counts per 100 ml and TC counts greater than 2,000 counts per 100 ml would fail to meet the required standards in the directive (European Community, 2006). Although the TC group includes the species *E. coli*, which is generally considered to be specific for faecal contamination, it also includes other genera such as *Klebsiella* and *Citrobacter* which are not necessarily of faecal origin and can emanate from alternative organic sources such as decaying plant materials and soils (WHO, 2006).

While some *E. coli* represent enteric pathogens (Savageau, 1983), other strains of this species can grow and maintain populations in the environment if the conditions are suitable (Byappanahalli and Fujjoka, 2004). Sources of *E. coli* include septic tanks, sewer lines, wastewater treatment plants, manure spreading on land, livestock and wildlife. These sources also contribute DOM and nutrient inputs. Despite advanced wastewater treatment efforts by water treatment companies, some UK bathing sites do not always produce full compliance with microbial standards (Crowther et al., 2002) due to other diffuse sources within catchments, resulting in a greater proportion of nonconformity due to agriculture. More than 150 different pathogens, associated with both environmental and human health risks can be found in livestock manure which can significantly increase bacterial loading to the subsurface, causing contamination within soils, groundwater and stream water (Gerba and Smith, 2005). The transport time and distance travelled by bacteria reaching the groundwater or streams depend on the rate at which bacteria are released from manure, the presence of preferential pathway networks within soil and the depth to the groundwater (Abuashour et al., 1994, Unc and Goss, 2003). The presence of TCs in surface or groundwater is usually considered evidence of recent faecal contamination, with *E. coli* remaining active for 16-45 days in the subsurface (Taylor et al., 2004).

This study aims to use a combination of inorganic chemistry, DOM fluorescence and culturable *E. coli* and TC counts to evaluate the potential sources and pathways of nutrients and contamination to a sand dune site designated for its international nature conservation importance, known to be affected by nutrients from the surrounding agricultural land (Rhymes et al. (2014)). Building on the previous study, a further year of bi-monthly sampling was carried out to separately assess the degree of potential contamination and likely sources of contaminants from a) off-site sources entering the site from streams, b) off-site sources entering the site via runoff/overland flow, c) groundwater flowing under the site, and d) on-site sources.

Materials and methods:

Field monitoring strategy

Aberffraw sand dune system is part of an internationally designated conservation site in the European Union Natura network, located on the southwest corner of the island of Anglesey in North Wales, UK (53°11'N, 4°27'W). It is designated for its dune habitats, in particular its dune slack wetlands and the rare plant and invertebrate species they support (Curreli et al., 2013). The site is in a low valley surrounded on three sides by agricultural land. The agricultural land is reseeded and fertilised pasture, used for sheep and cattle grazing, with feed stations on land immediately adjacent to the south-east dune site edge (Fig 1). Streams A and B (Fig 1) drain this heavily fertilised agricultural and both lead onto the site. Flow in stream A is episodic and flows primarily in winter, compared with the permanent and faster flowing stream B. Annual long term average rainfall at the site is 847mm (Stratford et al., 2013). There are a number of potential pathways by which nutrients and coliforms can enter the site, these include streams and ditches, surface runoff draining agricultural land and flowing onto the site, seepage of nutrients into the groundwater flowing under the site, and on-site sources such as grazing cattle and rabbits. Previous work has previously shown a nitrogen groundwater contamination gradient that extends into the site from the fertilised pastureland on the south east border with groundwater travelling in a south westerly direction (Fig 1) (Rhymes et al., 2014). To determine the nature and pathways of the contamination, measurements were made bimonthly for a 12 month period (i.e. 6 sample periods) across streams, ditches, standing surface water and groundwater in dune slacks. Stream samples were collected from two streams (A and B) entering the site (Fig 1). Samples were collected from upstream sampling points (A1 & B1) and downstream sampling points (A2 & B2) by dipping a clean collecting container into the surface flow. Groundwater samples within dune slacks were measured from fifteen groundwater monitoring piezometers across the site, installed to 2m depth. Four piezometers (Fig 1, triangles) aimed at looking at impacts from surface runoff. The remaining eleven aimed at evaluating potential gradients in the groundwater of water chemistry, natural fluorescence and TC and *E. coli* abundance with distance from the contamination sources on the south-east edge of the site (Fig 1, squares). Samples were collected from the top 10cm of the water table at each well using a sterilised pump and tubing, which was disinfected with Trigene and flushed three times with deionised water between samples into 250 mL sterile plastic bottles. During periods of inundation, for up to 4 months between November and February (Rhymes et al., 2014), when water tables were above ground level in certain slacks, samples of the standing water above the piezometer were taken. Groundwater depth was measured monthly at each piezometer. For fluorescence and *E. coli* measurements, sampling was conducted for 4 of the 6 sampling periods.

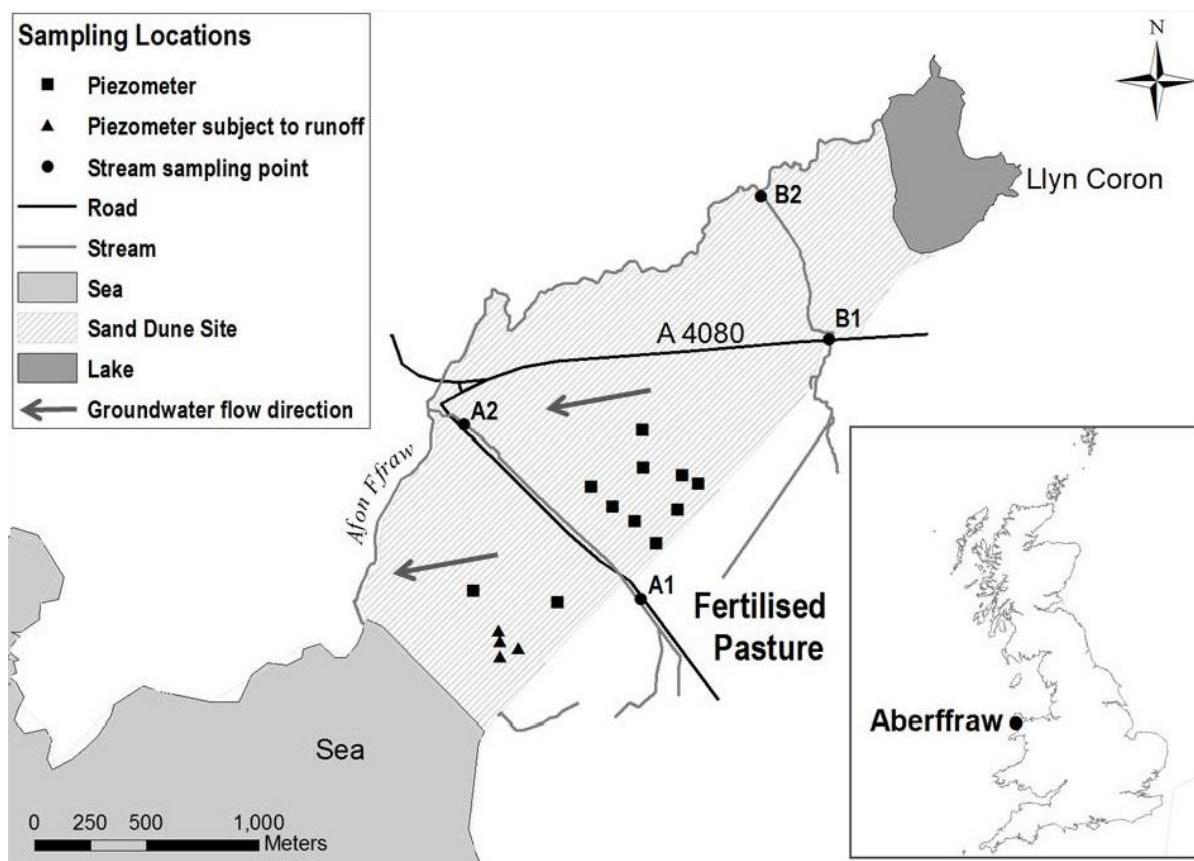


Fig 1. Map of Aberffraw dune system showing all piezometer and stream sampling points. Cross-hatched area represents designated site. The surrounding white area is agricultural land, predominantly pastureland. Redrawn from Ordnance Survey.

Water chemistry analysis

Samples from piezometers and streams were analysed within 24 hours of collection and stored in darkness at 5°C prior to chemical analysis. In the laboratory groundwater pH was recorded for each sample which was then filtered through 0.45 µm nylon syringe filter (Avonchem™). Dissolved inorganic anions (fluoride, chloride, nitrite, nitrate, phosphate and sulphate) and cations (sodium, ammonium, potassium, calcium and magnesium) were then quantified on an ion chromatograph (Metrohm, UK Ltd.). Dissolved inorganic nitrogen (DIN) was calculated as the sum of NO₃-N, NO₂-N and NH₄-N. Total nitrogen (TN) and total carbon were analysed by thermal oxidation on a thermalox TOC/TN analyser. Total inorganic carbon (TIC) was measured within a TIC-reactor on a thermalox. Dissolved organic nitrogen (DON) was calculated by the difference between TN and calculated DIN and Dissolved Organic Carbon (DOC) was calculated by the difference between TC and TIC.

Fluorescence analysis

All samples were filtered in the field using 0.45µm silver membrane filters (Steplitech) and stored in the dark at 5°C prior to analysis. Analysis took place 48 hours after collection at room temperature. Fluorescence measurements were obtained from a spectrophotometer (Variant Cary Eclipse) fitted with a xenon flash lamp using slit widths of 5nm, an integration time of 12.5ms and 700v voltage. Excitation wavelengths were varied from 200 to 400 nm in steps of 5nm and emission wavelengths from 280 to 500 nm in steps of 2 nm. Post processing was carried out using an R script described by Lapworth and Kinniburgh (2009) within the statistical package R. Absorbance was measured in a 1cm cuvette on a

UV-vis spectrophotometer (Varian Cary 50) at 1nm intervals from 800 to 200 nm and SUVA²⁵⁴ was calculated by dividing absorbance at 254 nm by DOC concentration (Weishaar et al., 2003). Absorbance measurements were scatter corrected employing the method of Blough et al. (1993). All fluorescence data was corrected for instrument effects to account for lamp output, and corrected for inner filter effects using the corrected absorbance data (Lakowicz and Geddes, 1991). The data were reported in standard Raman units, which normalises the intensity by the area under the Raman peak between emission wavelengths 380-410 for the excitation wavelength of 348 nm.

Enumeration of TC and *E. coli*

Water samples were processed within 6 hours of collection. *E. coli* and TC counts in water samples were determined in duplicate by filtering 20 ml of water through 0.2 µm cellulose nitrate filters (Whatman). Subsequently, cellulose nitrate filters were aseptically transferred onto Harlequin™ *E. coli*/Coliform Medium (Lab M). Culture plates were incubated for 22 hours at 37°C prior to colony counting: TCs generated purple colonies and *E. coli* produced blue colonies Harlequin™ *E. coli*/Coliform Medium.

Statistical analysis:

All statistical analysis was performed using minitab v.16. Differences in stream water chemistry concentrations, fluorescence concentrations and *E. coli* and TC counts between upstream (A1, B1) and downstream (A2, B2) sampling points in two streams, A and B, were assessed using ANCOVA (Stevens, 1982), where the date of sample collection was used as a covariate to account for seasonal variation.

In order to test for statistical differences in chemical, fluorescence and *E. coli* and TC count variables between streams, runoff and underlying groundwater gradients, annual means of all variables were analysed by grouping piezometers into three classes based on their distance from the south-east site edge (0-150 m, 150-300 m, 300-450 m, excluding piezometers which were subject to run-off), grouping piezometers subject to runoff for March samples alone (see explanation below) and annual mean upstream sampling points from streams A and B (A1 & B1). Statistical tests used analysis of variance. Data that proved not normally distributed (Kolmogorov-Smirnov test) were transformed using a Johnson's transformation, which transforms the data to follow a normal distribution using Johnson distribution system.

In order to assess runoff inputs separately from any contributions from on-site sources, samples from piezometers subject to runoff (Fig 1 triangles) were compared with all other piezometers (Fig 1 squares) for samples collected in March, as this was the only month where all piezometers were subject to groundwater flooding. An ANCOVA (Stevens, 1982) was carried out on these two groups for all water chemistry, *E. coli* and TC counts and fluorescent spectroscopy variables, with distance from the south-east site edge as a covariate to account for potential underlying gradients in water chemistry due to other sources. Data that proved not normally distributed (Kolmogorov-Smirnov test) were transformed using a Johnson's transformation.

In order to assess underlying input gradients via the groundwater into the site, whilst accounting for runoff inputs, we separately analysed annual means of variables for all piezometers that were not subject to runoff (Fig 1 squares). Relationships between annual means of all measurements with distance from

the south-east site edge were investigated using linear regression. Data that proved not normally distributed (Kolmogrov-Smirnov test) were transformed using a Johnson's transformation.

Results:

When comparing the water chemistry, *E. coli* and TC counts and fluorescence among the main sources (Table 1), nitrate and DIN concentrations in runoff and streams were significantly higher than those found in groundwater samples from the 150-300 m and 300-450 m distance classes. There was no significant difference in nitrate concentrations between the three distance classes. However, significantly higher concentrations of DIN were observed in the 0-150 m class closest to the south-east site edge compared with the 300-450 m class. *E. coli* counts in the upstream sampling points of streams were significantly higher by an order of magnitude than in the runoff samples and in the groundwater at all distance classes (Table 1). TCs in streams were significantly higher than in the slacks subject to runoff, but were not significantly different from groundwater. There were no significant differences between sampling locations for DOC, Phosphate, fulvic like, tryptophan like, TRP:FA and SUVA²⁵⁴. All fluorescent TRP:FA ratios measured within surface waters, groundwater and streams throughout the year did not exceed 1 R.U. and are described as uncontaminated drainage waters (Naden et al., 2010).

Table 1. Summary of annual mean water chemistry, *E. coli* and TC counts and fluorescence concentrations and counts for upstream sampling points for two streams (A and B), mean standing water for flooded slacks subject to runoff in March and for annual mean groundwater and standing water for distance classes (categorised piezometers located 0-150 m, 150-300 m and 300-450 m away from the south east site edge, excluding piezometers subject to run off). Values for each variable are expressed as mean \pm standard error. Significant differences among classes are shown in bold; values with the same letter are not significantly different to each other.

Variable		Stream	Groundwater and standing water			
			Run off	(Distance from site edge, m)		
				0-150	150-300	300-450
Water Chemistry (mg/L)	Nitrate	20.821 \pm 9.763^A	7.519 \pm 1.556^A	3.197 \pm 1.516^{AB}	0.033 \pm 0.019^B	0.018 \pm 0.007^B
	DIN	4.720 \pm 2.209^A	1.702 \pm 0.352^A	0.756 \pm 0.346^{AC}	0.050 \pm 0.019^{BC}	0.017 \pm 0.003^B
	DON	0.987 \pm 0.459^A	0.658 \pm 0.042^{AB}	0.430 \pm 0.076^{AB}	0.322 \pm 0.088^B	0.249 \pm 0.034^B
	DOC	8.492 \pm 13.903	24.600 \pm 13.903	14.250 \pm 0.966	7.789 \pm 4.878	8.602 \pm 1.863
	Phosphate	0.750 \pm 0.654	0.009 \pm 0.003	0.020 \pm 0.003	0.012 \pm 0.001	0.015 \pm 0.005
Bacterial Counts (Log 10 Counts per 100ml)	<i>E. coli</i>	3.458 \pm 0.033^A	0.000 \pm 0.000^B	0.151 \pm 0.151^B	0.403 \pm 0.242^B	0.285 \pm 0.133^B
	TC	4.087 \pm 0.197^A	2.559 \pm 0.169^B	2.863 \pm 0.083^{AB}	2.793 \pm 0.355^{AB}	2.641 \pm 0.431^{AB}
Fluorescence (R.U.)	Fulvic like	1.423 \pm 0.092	1.491 \pm 0.003	1.321 \pm 0.048	1.367 \pm 0.078	1.251 \pm 0.041

	Tryptophan like	1.141 ± 0.030	1.167 ± 0.002	1.114 ± 0.015	1.123 ± 0.024	1.097 ± 0.016
	TRP:FA	0.810 ± 0.036	0.782 ± 0.001	0.844 ± 0.019	0.838 ± 0.027	0.888 ± 0.017
Absorbance (L mg ⁻¹ m ⁻¹)	SUVA ²⁵⁴	0.043 ± 0.011	0.061 ± 0.046	0.018 ± 0.003	0.020 ± 0.003	0.015 ± 0.005

Stream nutrient and bacterial attenuation

Upstream annual mean nitrate and DIN concentrations are significantly higher in stream A than in stream B, however *E. coli* counts are alike for both A and B streams for both upstream and downstream sampling points. Annual mean nitrogen concentrations upstream of streams entering the site are high at all stream sampling points (e.g. annual mean 12 mg/L of nitrate and 2.6 mg/L of DIN at B1) but are very high in A1, which drains from the south-east site edge into the site, where concentrations reached a maximum of 39 mg/L of nitrate in January. In stream A, the annual mean concentrations and counts for nitrate, DIN and *E. coli* were significantly higher upstream (A1) than those downstream (A2) (Fig. 2). By contrast, concentrations of nitrate and DIN in stream B (B1) did not decrease downstream to B2. No significance was found for fluorescence variables for all stream sampling points, TRP:FA mean concentrations were A1= 0.217 ± 0.034 R.U., A2= 0.280 ± 0.042 R.U., B1= 0.457 ± 0.203 R.U. and B2= 0.268 ± 0.042 R.U. Total nitrogen concentrations showed the same significant pattern as nitrate concentrations for all stream sampling points.

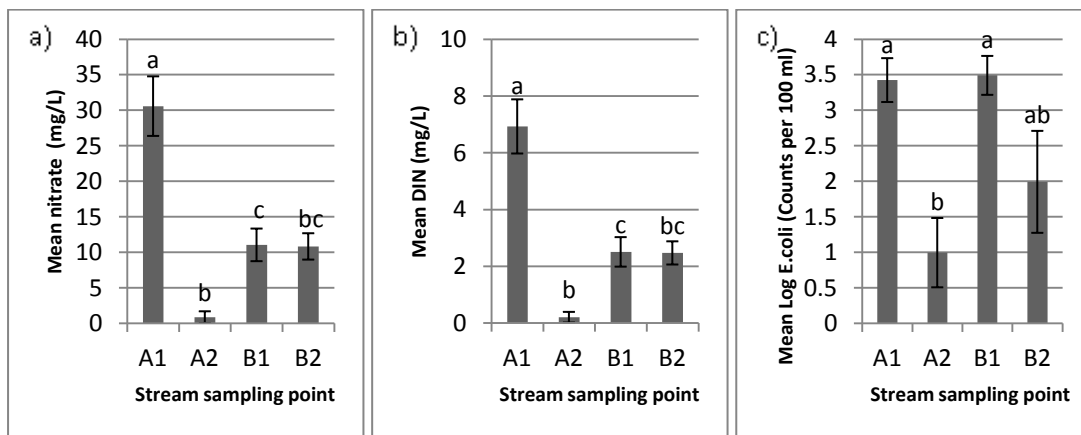


Fig 2. Annual mean concentrations for nutrients and *E. coli* counts of two streams (A and B) entering the site at upstream and downstream sampling points, showing: a) Nitrate b) DIN c) Log *E. coli* counts. Letters denote significant differences between stream sampling points A1 & B1 –upstream, A2 & B2 downstream – see Fig 1.

Run off input

In order to assess the contribution of off-site sources of contamination (cattle feed, overnight dunging, manure or slurry spreading) separately from any contribution by on-site sources (rabbits, dunging of

cattle while grazing on-site), slacks only subject to groundwater flooding (Fig 1 Squares) were compared with those experiencing groundwater flooding in addition to run off from neighbouring fields (Fig 1 Triangles), during a period when both sets of piezometers experienced surface inundation (Table 2). Significantly higher concentrations of nitrate and DIN were observed in those slacks exposed to run off. However, there was no significant difference for DON, DOC, *E. coli* and TC counts, fluorescent variables and all other variables measured (Table 2).

Table 2. Summary of selected chemical, fluorescent and *E. coli* and TC count variables for piezometers subject to both run off and groundwater flooding and wells subject to groundwater flooding alone in March. Values for each variable are expressed as mean \pm standard error. Significant differences between groups of slacks are shown in bold and denoted by letters.

Variable		Slacks subject to:	
		Run off and groundwater flooding	Groundwater flooding
Water chemistry (mg/L)	Nitrate	7.519 \pm 1.556^A	0.676 \pm 0.386^B
	DIN	1.702 \pm 0.352^A	0.181 \pm 0.0920^B
	DON	0.658 \pm 0.042	0.505 \pm 0.072
	DOC	11.100 \pm 4.700	19.117 \pm 2.023
	Phosphate	0.014 \pm 0.009	0.024 \pm 0.008
Bacterial counts (Log ₁₀ Counts per 100ml)	<i>E. coli</i>	0.000 \pm 0.000	0.841 \pm 0.351
	TC	3.036 \pm 0.301	3.370 \pm 0.086
Fluorescence spectroscopy (R.U.)	Fulvic like	1.491 \pm 0.003	1.515 \pm 0.074
	Tryptophan like	1.167 \pm 0.002	1.172 \pm 0.023
	TRP:FA	0.782 \pm 0.001	0.785 \pm 0.024
Absorbance (L mg ⁻¹ m ⁻¹)	Suva ²⁵⁴	0.061 \pm 0.046	0.018 \pm 0.003

Underlying gradients of nitrogen input via groundwater and on-site inputs

In order to assess underlying input gradients via the groundwater into the site, whilst accounting for run off inputs, we separately analysed annual means of variables for all piezometers that were not subject to run off against distance from potential source area at the south-east site edge (Figure 3). There were significant gradients of declining nitrate, DIN and DON concentrations into the site, away from their likely source at the south-east site edge (Significant negative regression Fig 3; a) Nitrate Coef = -0.005, b) DON Coef= -0.001 and c) DIN Coef= -0.), with nitrate and DIN decreasing very strongly. A trend of linear decline was apparent for DOC concentrations but this was not significant (Fig 3d). Figure 4 shows the spatial pattern of *E. coli* and TC counts and tryptophan-like and fulvic-like fluorescence. Counts of *E. coli* (Fig 3e and 4b) and TC (Fig 4a) observed across the site showed no correlation with distance from the south-east site edge, (Log *E. coli* counts/100 mL; max= 4.6, min= 0.00 and Log TC counts/100mL; max= 4.85, min= 0.00). Tryptophan like fluorescence and fulvic like fluorescence (Fig 3f and 3g) also showed no correlation with distance from the south-east south edge. However the TRP:FA ratio (Fig 3h) showed a strong positive significant trend for increasing TRP:FA ratio away from the south-east site edge,

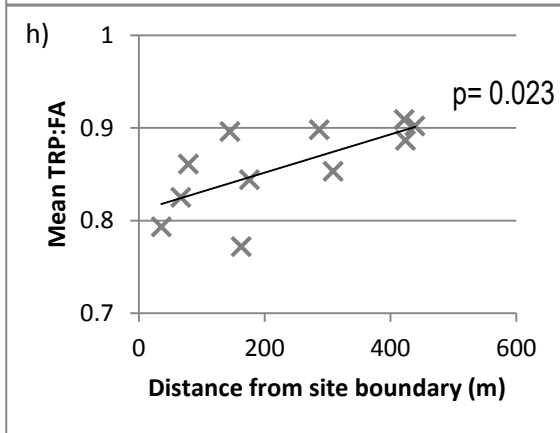
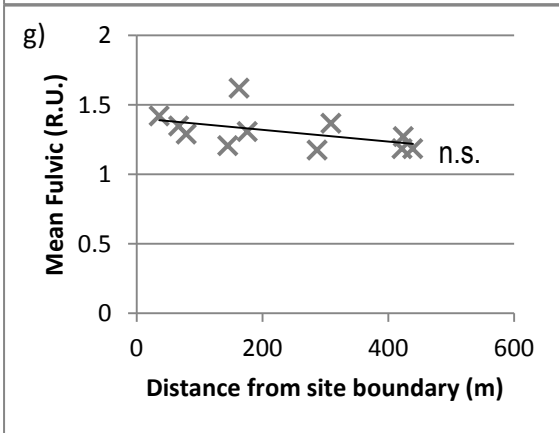
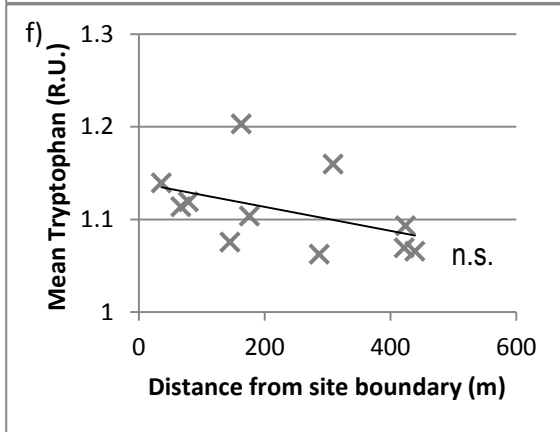
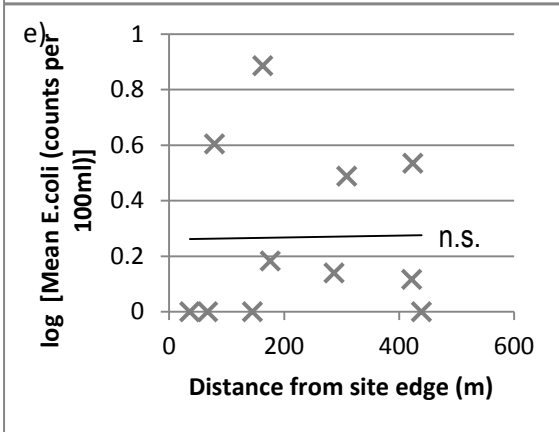
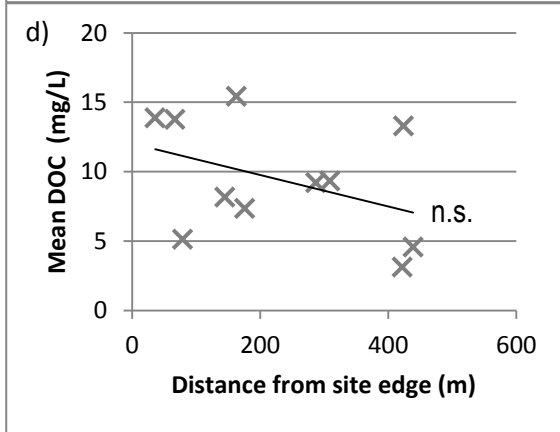
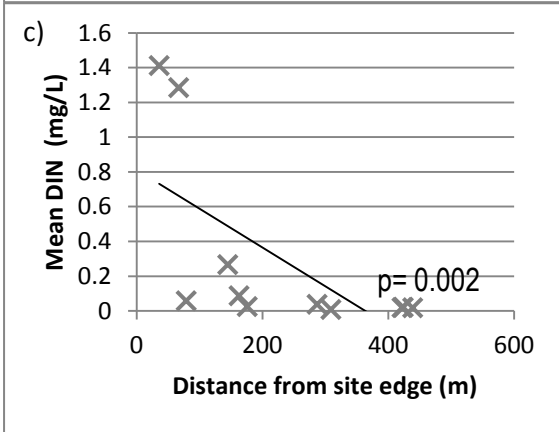
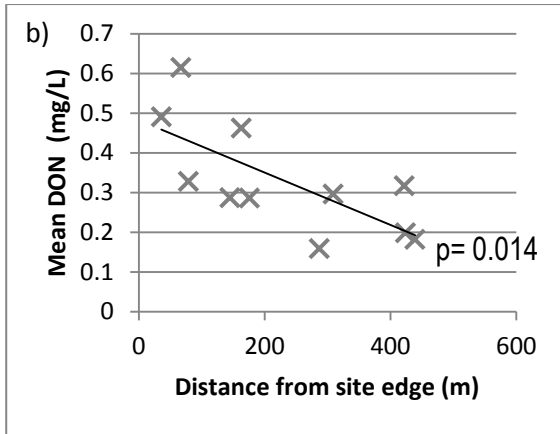
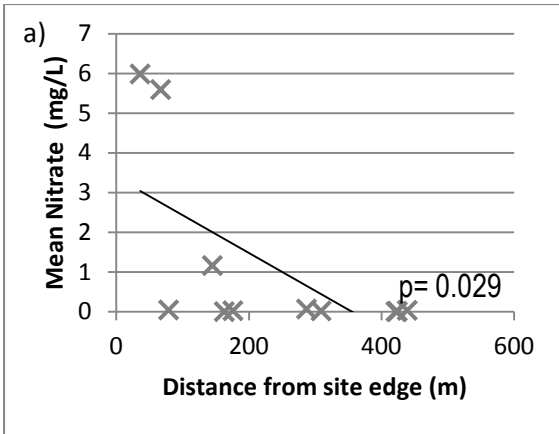


Fig 3. Relationships between annual mean groundwater a) Nitrate b) DON c) DIN d) DOC e) *E. coli* f) Tryptophan-like g) Fulvic-like h) TRP:FA with distance from south-east site edge. Trendline for all variables are linear.

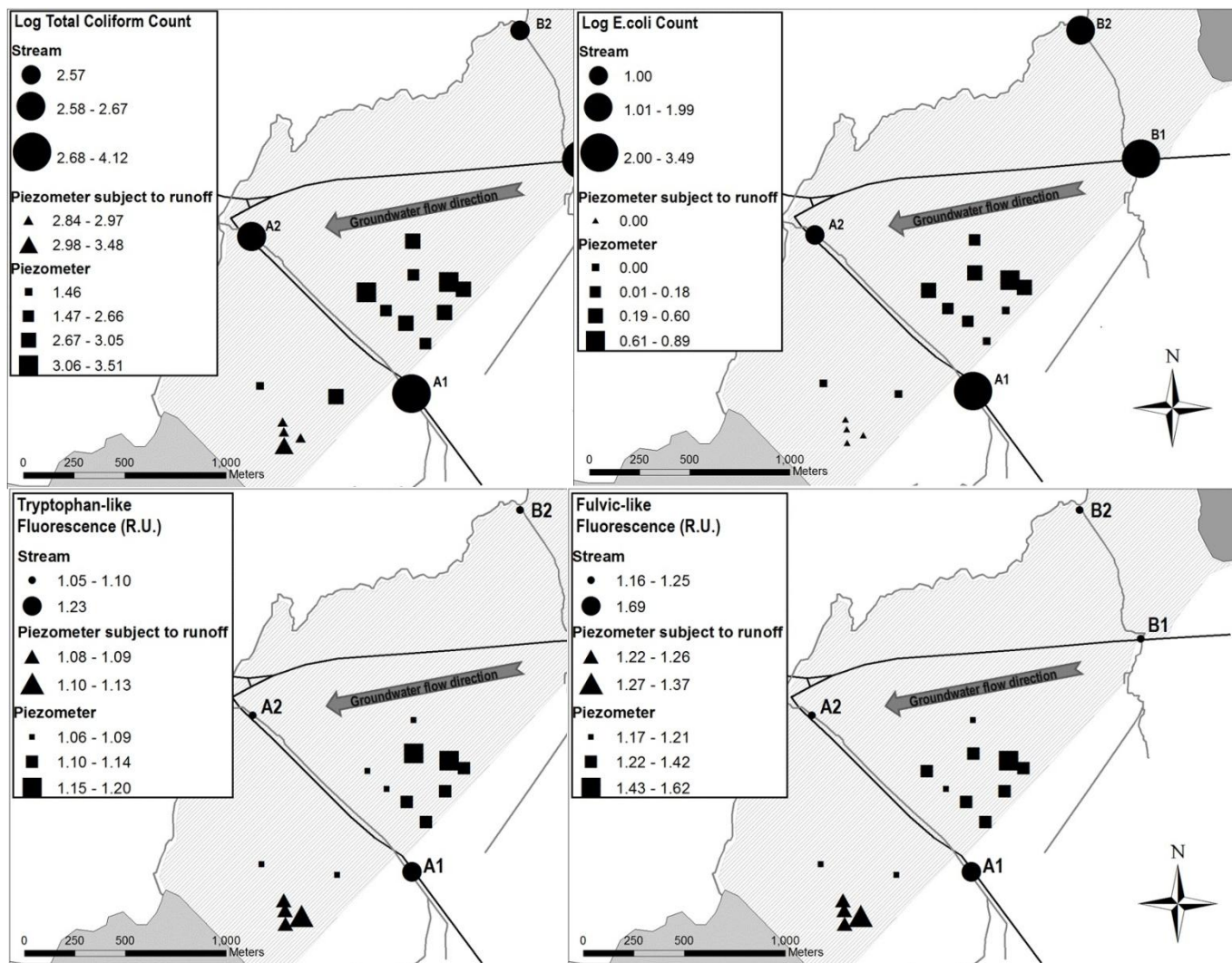


Fig 4. Spatial variation of a) Log *E. coli* counts/100 mL, b) Log total coliform counts/100 mL, c) tryptophan-like fluorescence (R.U.) and d) fulvic-like fluorescence (R.U) for streams (annual mean), piezometers subject to runoff (March counts and concentrations) and piezometers (annual mean).

Discussion:

In this study we show that a combined analysis of nutrient concentrations, fluorescence and microbial abundance can help identify potential sources and pathways of nutrients and *E. coli* and TC inputs impacting a wetland site of international nature conservation importance. The multiple pathways and the fate of nutrients and *E. coli* and TC to the site are summarised in Figure S1 in the Supplementary Material.

Off-site sources (fertiliser, cattle dung, slurry and fertilisers applied to fields)

Streams

Streams A and B have similar annual mean *E. coli* abundances as they enter the site, suggesting that both streams have similar faecal inputs from grazers on adjacent pastureland. By contrast, nitrate concentrations in Stream A, which drains the pastureland and flows onto the sand dune site, are significantly higher than those in stream B and previous studies have shown they can exceed the 50 mg/L threshold for designation of a nitrate vulnerable zone by the UK Environment Agency (Rhymes et al., 2014, Environment Agency, 2012). This implies that stream A has additional N inputs compared to stream B, these are likely to be from fertilisers leaching from the steep sloped pastureland adjoining the stream. This contrast with the fluorescence results where the lack of significant difference in TRP:FA ratio between the two streams suggesting a common contamination source from animal dung, with differences in nutrient loading due to fertiliser inputs only.

The attenuation of nitrate concentrations in stream A is probably caused by a combination of processes, including in-stream microbial denitrification, plant uptake, seepage through the river bank, dilution during high groundwater levels and transient storage (Mulholland et al., 2008). The lack of nutrient attenuation in stream B may be due to the absence of nitrophilous species such as *Phragmites australis* (Stamati et al., 2010) and the faster stream flow in stream B which reduces the water-sediment contact (Peterson et al., 2001) and reduces the ability for microbes to assimilate nitrogen from the water column (Grimm and Fisher, 1989). Similarly *E. coli* counts decrease from upstream to downstream sampling points in stream A but not stream B. Studies have shown that within sediment there are higher populations of TCs than the overlying water (Smith et al., 2008) as sediments serve as a hospitable environment for bacterial survival due to the availability of organic matter (Jamieson et al., 2005) suggesting that *E. coli* may be being deposited into the stream and incorporated within the sediment. Subsequently during storm events bacteria can be re-suspended into the water column and continue to flow downstream (Jeng et al., 2005), posing a potential threat to bathing waters on the sandy beach at Aberffraw at the mouth of the river Ffraw since mean *E. coli* colonies within both streams exceed 2,000 colonies per 100 ml which would fail to meet the required standards for the EU bathing water directive (2006).

Surface runoff

The nature of the nitrate contamination contributing via runoff is also likely to be due to applied fertilisers on the south-east pastureland, rather than nutrients from slurry and dung. Nitrate concentrations are eleven times higher in slacks subject to run off and groundwater flooding compared to piezometers subject to groundwater flooding alone, whereas the TRP:FA fluorescence ratios were <1 R.U. which are described as uncontaminated drainage waters compared to described slurry TRP:FA fluorescence ranging from 2-5 R.U. (Naden et al., 2010). In support of this, negligible *E. coli* and TC counts were observed in slacks subject to runoff which would have been expected if the contamination resulted from slurry application (Thurston-Enriquez et al., 2005). Surface runoff events are sporadic as they are caused by heavy periods of rainfall, nevertheless while nutrient concentrations are high during runoff events and in the subsequent standing surface water, the concentrations in groundwater return to low concentrations once the runoff ceases. This may be a result of denitrification caused by the increased availability of nitrate and anaerobic conditions (Mulvaney et al., 1997), or due to plant and microbial uptake highlighting the function of dune systems in filtering nutrients. However, the initial

nitrate and DIN concentrations in the standing water are much higher than the levels of 0.2 mg DIN L⁻¹ above which biological effects have been determined previously at the site (Rhymes et al. 2014), suggesting adverse impacts on the site due to nutrients from this source. In addition, the total flux of nitrogen entering the site as a result of these runoff events is also unknown. Calculating this input may help harmonise dose-related critical load approaches to damage (Bobbink and Hettelingh, 2010) with concentration based methods used in aquatic systems (Camargo and Alonso, 2006).

Groundwater

Nutrients, likely to be from ammonium nitrate fertiliser, are entering the site by leaching through sandy agricultural soils with a low water holding capacity (Skiba and Wainwright, 1984), and subsequently flowing under the site via the groundwater. As a result a nitrate, DIN and DON groundwater gradient was found independently of runoff influence, confirming that the gradients of elevated nutrients in groundwater and soils observed in an earlier study were not due to surface flooding (Rhymes et al., 2014). There was no gradient in *E. coli* and TC counts within the groundwater under the site, suggesting that the higher concentrations of *E. coli* and TCs originating in the south-east pasture and observed in the streams are probably filtered out by the sandy soils during recharge transit in the subsurface before reaching the site (Price et al., 2013).

On-site sources (rabbits, cattle dung)

E. coli and TCs were found across the site at low levels, but showed no relationship with the distance from the south-east site edge. Similarly, the tryptophan like fluorescence showed no relationship with the distance from the south-east site edge. This suggests that the main source of groundwater *E. coli*, and TCs, derive from on-site cattle and rabbits. Therefore, in addition to streambed seepage, run off and underlying groundwater nutrient inputs, there are nutrient and *E. coli* and TC inputs from on-site grazers such as cattle and rabbits. Despite these small-scale on-site inputs, all piezometers across the site meet the mandatory bathing water directive standards of 2,000 *E. coli* counts per 100 mL.

Conclusions

The combination of chemical, fluorescent and microbial techniques has helped identify potential nutrient sources from fertilisers and grazers (Baker, 2002). The findings of this study suggest nutrients are being attenuated and processed within the site thereby providing a valuable ecosystem service. However at the same time, the influx of nutrients is likely to have an adverse effect on the dune slack ecology, with impacts on plant community composition (Rhymes et al., 2014). The analysis of surface waters, slacks and piezometers across the site has allowed the differentiation between the input pathways of streams, run off events, underlying groundwater nutrient gradients and on-site grazing inputs. While the study was able to distinguish multiple pathways, the full potential of the techniques to differentiate between livestock sources (e.g. sheep, cattle, pigs) was not explored in this study. This combination of techniques provides an approach which could allow for a detailed understanding of nutrient contamination sources and pathways relatively cheaply. Such information is key for designing successful management plans to reduce the inputs of contaminants which might be having detrimental effects on sites of conservational value. It could also be implemented for other applications such as tracking faecal sources within bathing waters and fisheries zones, as currently the standard

enumeration of FIB does not distinguish between human or other animal sources of contamination, and methods that do so are expensive (Field and Samadpour, 2007).

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